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## **cAMP-Binding Protein Epac Induces Cardiomyocyte Hypertrophy**

Eric Morel, Andrea Marcantoni, Monique Gastineau, Rikke Birkedal, Francesca Rochais, Anne Garnier, Anne-Marie Lompré, Grégoire Vandecasteele and Frank Lezoualch

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# cAMP-Binding Protein Epac Induces Cardiomyocyte Hypertrophy

Eric Morel, Andrea Marcantoni, Monique Gastineau, Rikke Birkedal, Francesca Rochais, Anne Garnier, Anne-Marie Lompré, Grégoire Vandecasteele, Frank Lezoualc'h

**Abstract**—cAMP is one of the most important second messenger in the heart. The discovery of Epac as a guanine exchange factor (GEF), which is directly activated by cAMP, raises the question of the role of this protein in cardiac cells. Here we show that Epac activation leads to morphological changes and induces expression of cardiac hypertrophic markers. This process is associated with a  $\text{Ca}^{2+}$ -dependent activation of the small GTPase, Rac. In addition, we found that Epac activates a prohypertrophic signaling pathway, which involves the  $\text{Ca}^{2+}$  sensitive phosphatase, calcineurin, and its primary downstream effector, NFAT. Rac is involved in Epac-induced NFAT dependent cardiomyocyte hypertrophy. Blockade of either calcineurin or Rac activity blunts the hypertrophic response elicited by Epac indicating these signaling molecules coordinately regulate cardiac gene expression and cellular growth. Our results thus open new insights into the signaling pathways by which cAMP may mediate its biological effects and identify Epac as a new positive regulator of cardiac growth. (*Circ Res.* 2005;97:0-0.)

**Key Words:** cAMP ■ guanine nucleotide exchange factor ■ small G protein ■ transcription factor

In the heart, cyclic adenosine 3',5'-monophosphate (cAMP) regulates many physiological processes such as contractility and relaxation. Classically, these effects are attributed to activation of hyperpolarization-activated cyclic nucleotide-gated channels and protein kinase A (PKA) by cAMP.<sup>1</sup> The recent discovery of Epac as proteins which are directly activated by cAMP has broken the dogma surrounding cAMP and PKA.<sup>2-4</sup> Epac proteins are guanine nucleotide exchange factors (GEFs) that bind cAMP with affinities similar to that of the regulatory subunit of PKA.<sup>2,3</sup> They have been shown to function as GEFs for the Ras-like small GTPases Rap1 and Rap2 and are directly activated by cAMP in a PKA independent manner.<sup>4</sup> There are two isoforms of Epac, Epac 1 and Epac 2 both consisting of a regulatory and a catalytic region.<sup>2,3</sup> Epac 2 has an additional cAMP binding domain that is dispensable for cAMP-induced Rap activation.<sup>5</sup> After cAMP binding, Epac catalyzes the exchange of GDP for GTP on the small GTPases Rap, allowing interaction with their target effectors.<sup>6</sup> Recent studies indicate that Epac is involved in cell adhesion,<sup>7,8</sup> neurite extension,<sup>9</sup> and regulates insulin secretion and the amyloid precursor protein processing.<sup>10,11</sup> To date the role of Epac in the heart is unknown.

Among the superfamily of small G proteins, the Rho family, which includes Rho, Rac, and Cdc42, has attracted much interest for they have been shown to play key roles in the generation of cytoskeletal structures.<sup>12</sup> Indeed, Rho is

important for the formation of stress fibers and focal adhesions in fibroblasts, whereas Rac and Cdc42 are involved in the regulation of more dynamic structures such as membrane ruffles, lamellipodia and filopodia.<sup>12</sup> Several studies have pointed out the role of Rho proteins in the development of cardiomyocyte hypertrophy.<sup>13</sup> For instance, two potent hypertrophic stimuli, endothelin 1 (ET-1) and phenylephrine (PE), induce rapid activation of endogenous Rac in neonatal cardiomyocytes.<sup>14</sup> In addition, adenoviral infection of cardiomyocytes with a constitutive active form of Rac (Rac<sup>G12V</sup>) increases protein synthesis and promotes morphological changes associated with myocyte hypertrophy.<sup>15</sup> In vivo evidence for the role of Rho proteins in cardiac hypertrophy came from transgenic mice specifically expressing Rac<sup>G12V</sup> in the heart. These mice develop a dilated cardiomyopathy associated with deregulation of cardiomyocyte focal adhesions.<sup>16</sup> These data suggest that Rho proteins, especially Rac control hypertrophic response and are likely to be involved in cardiac remodeling, and the pathogenesis of cardiomyopathy characterized by cellular enlargement.

Recently, we have provided experimental evidence that Epac stimulates the activity of the small GTPase, Rac, in a cAMP-dependent but PKA-independent manner in neuronal cells.<sup>11</sup> These observations combined with the high expression of Epac in the heart<sup>2,3</sup> prompted us to focus our research on the potential role of Epac in cardiomyocyte hypertrophy.

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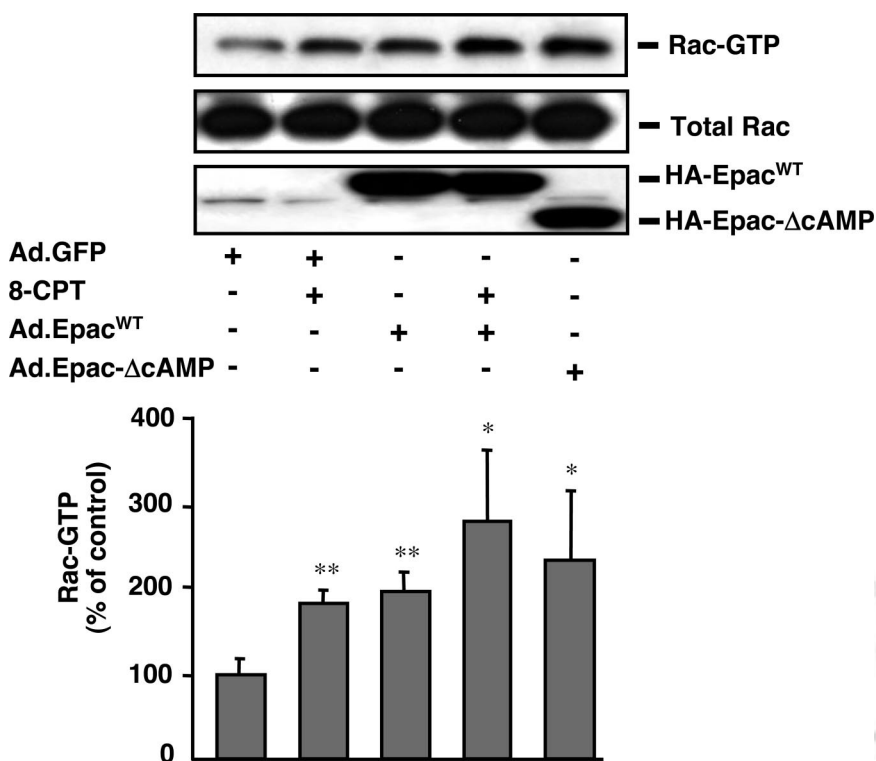
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**Figure 1.** Epac activates the small G protein Rac in primary ventricular cardiomyocytes. Cardiomyocytes were infected with either Ad.GFP as a control or with Ad.Epac<sup>WT</sup> or Ad.Epac-ΔcAMP as described in Materials and Methods. Two days after infection, cells were treated or not for 10 minutes with the selective activator of Epac, 8-CPT ( $10^{-6}$  mol/L). Amounts of Rac-GTP were determined by pull-down experiments. A control for total Rac expression (total lysates) is shown. The upper panel shows a typical immunoblot. Expression of recombinant proteins was determined by Western blot using an anti-HA antibody. The lower panel shows means  $\pm$  SEM of 3 independent experiments. Results are expressed as fold activation of control cells. \* $P < 0.05$ , \*\* $P < 0.01$  compared with control.

Here we found that Epac stimulates the activity of the small GTPase, Rac, and increases the expression of hypertrophic gene markers in primary cardiac myocytes. Furthermore, we show that Epac induces cardiomyocyte hypertrophy. This process is associated with the activation of Rac and the calcineurin/NFAT signaling pathway, which coordinately regulates cell growth and gene expression. Altogether, these findings identify the cAMP-binding protein, Epac, as a new positive regulator of cardiac growth.

## Materials and Methods

### Adenoviral Infection

Bicistronic adenoviruses (Ad5) bearing either Epac<sup>WT</sup> or EpacΔcAMP under the control of a cytomegalovirus promoter and green fluorescent protein (GFP) under internal ribosomal entry site control were constructed and amplified at the Genethon Center of Evry (France). Adenoviruses encoding VIVIT, a selective peptide inhibitor of calcineurin-mediated NFAT activation, and Rac were provided by Drs S. Kraner and C. Norris (University of Kentucky) and T. Finkel (Cardiology Branch, National Heart, NIH, Bethesda, Md), respectively. One day after plating, cardiomyocytes were incubated for 2 hours with recombinant adenoviruses. After removal of the virus suspension, cells were replaced in maintenance medium for 2 days and then stimulated with the different drugs. Viruses were used at a multiplicity of infection (MOI) of 100.

### Plasmid Constructs and Transfection

The plasmid constructs were generously provided by the following: the rat ANF promoter fused to the luciferase reporter gene (ANF-Luc) by Dr K. Knowlton, Luciferase reporter genes linked to promoters for skeletal muscle  $\alpha$ -actin (SkM- $\alpha$ -actin-Luc) and serum response element-regulated *c-fos* (*c-fos*-SRE-Luc) by Dr M. D. Schneider, Epac1 plasmid constructs by Drs J. Bos and X. Cheng. The luciferase reporter plasmid driven by four NFAT consensus binding sites (NFAT-Luc) was obtained from Stratagene. Transient transfection experiments were performed with Lipofectamine 2000

(Invitrogen Life Technologies, France) in optimum medium in the presence of 1  $\mu$ g of the various plasmid constructs according to the manufacturers' instructions.

### Rac Activation Assay

Rac pull-down experiments were performed using a GST fusion protein containing the Cdc42/Rac Interactive Binding Domain (CRIB) of p21-activated kinase (PAK) exactly as previously described.<sup>11</sup>

### Statistical Analysis

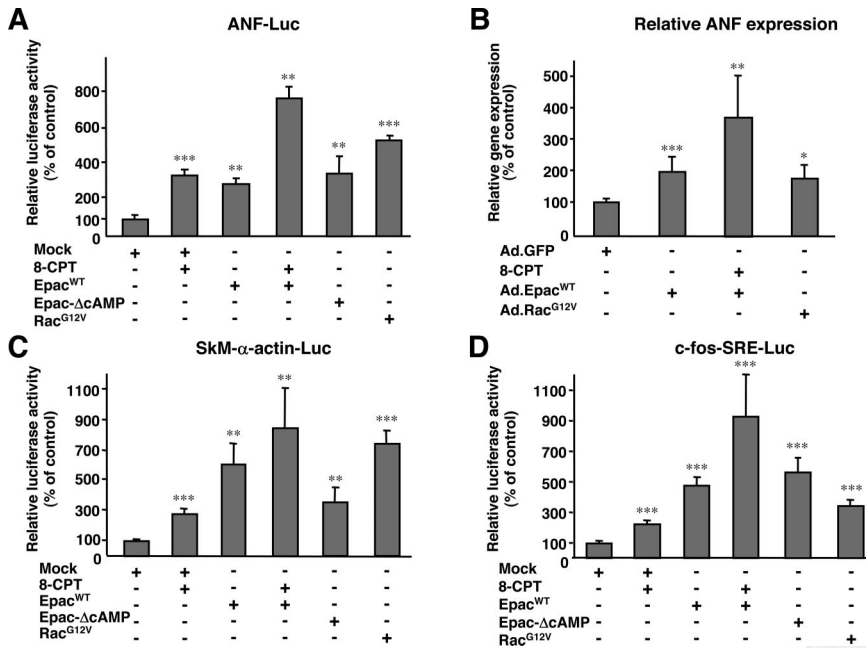
Results are expressed as means  $\pm$  SEM. Differences between groups have been analyzed by one-way ANOVA followed by unpaired Student *t* test. Differences were considered significant at  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ .

For a description of other methods, see the expanded Materials and Methods, available online at <http://circres.ahajournals.org>.

## Results

### Epac Activates the Small G Protein Rac in Cardiomyocytes

Activation of endogenous Epac with a selective activator of this GEF, 8-pCPT-2'-O-Me-cAMP (8-CPT), which does not activate PKA<sup>17</sup> increased Rac activation in rat cardiac myocytes (Figure 1). Similarly, infection of cardiomyocytes with an adenovirus encoding Epac1<sup>WT</sup> (Ad.Epac<sup>WT</sup>) significantly enhanced Rac GTP-loading compared with control cells infected with GFP (Figure 1). Rac activation was further increased when cells infected with Ad.Epac<sup>WT</sup> were treated with 8-CPT (Figure 1). An adenovirus bearing a constitutive activated form of Epac1 (Ad.Epac-ΔcAMP)<sup>2</sup> also induced Rac activation (Figure 1). Altogether, these results demonstrate that recombinant and native Epac increase the amount of Rac-GTP in cardiomyocytes.



**Figure 2.** Epac stimulates a hypertrophic pattern of gene expression. A, C, and D, Neonatal cardiomyocytes were transfected with ANF-Luc, SkM- $\alpha$ -actin-Luc or c-fos-SRE-Luc and Epac<sup>WT</sup>, Epac- $\Delta$ cAMP, Rac<sup>G12V</sup>, or the empty vector (mock) as control and treated or not with 8-CPT ( $10^{-6}$  mol/L). Two days after transfection, cells were assayed for Luc activity. Results are expressed as percentage activation of control. Results are means  $\pm$  SEM from 3 independent experiments performed in triplicates. B, Epac induces expression of ANF mRNA. Cardiomyocytes were infected with Ad.Epac<sup>WT</sup>, Ad.Rac<sup>G12V</sup>, or Ad.GFP (control) and stimulated or not with 8-CPT ( $10^{-6}$  mol/L) for 2 days. ANF mRNA expression was determined by quantitative PCR. Values are expressed relative to the ANF/GCB ratio and results were normalized to control for each experiment. Results are presented as the mean  $\pm$  SEM of 3 independent experiments performed in duplicates. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with control.

### Epac Increases the Expression of Hypertrophy Gene Markers

As Rac has been found to be involved in cardiac myocyte hypertrophy,<sup>15,16</sup> we next tested the potential involvement of Epac in this process. Re-expression of embryonic genes and transient activation of immediate early genes are frequently used indexes of myocyte hypertrophy.<sup>18</sup> The ability of Epac to stimulate gene expression was determined using luciferase (Luc) constructs under the control of promoters for ANF, SkM  $\alpha$ -actin, and the c-fos-SRE. Figure 2A shows a three-fold activation of the ANF-Luc reporter gene in neonatal cardiomyocytes stimulated with 8-CPT compared with control cells. Transient transfection of Epac<sup>WT</sup> or Epac- $\Delta$ cAMP increased the basal level of ANF-Luc activity (Figure 2A). The effect of Epac<sup>WT</sup> on ANF-Luc activity was further increased by the application of 8-CPT (Figure 2A). Rac<sup>G12V</sup> mimicked the effect of Epac on ANF-Luc activity (Figure 2A). Next, to analyze the effect of Epac on ANF mRNA content in cardiac myocytes, we used Ad.Epac<sup>WT</sup> to maximize the expression of this GEF in primary cardiomyocyte. Consistent with the effect of Epac on ANF-Luc reporter gene, endogenous expression of ANF mRNA was significantly increased in cardiomyocytes infected with Ad.Epac<sup>WT</sup> and stimulated or not with 8-CPT, as compared with control cells (Figure 2B). Similar results were obtained with an adenovirus expressing Rac<sup>G12V</sup> (Ad.Rac<sup>G12V</sup>) (Figure 2B). In addition, when cotransfection experiments were performed with SkM- $\alpha$ -actin-Luc or c-fos-SRE-Luc, 8-CPT, Epac<sup>WT</sup>, Epac- $\Delta$ cAMP, or Rac<sup>G12V</sup> significantly increased Luc activity compared with control cells (Figure 2C and 2D). The effect of Epac<sup>WT</sup> on Luc activity was further increased by the application of 8-CPT (Figure 2C and 2D).

### Epac Increases Cardiomyocyte Size and Sarcomeric Organization

Further studies were undertaken to determine the effects of Epac on other features of the hypertrophic program such as

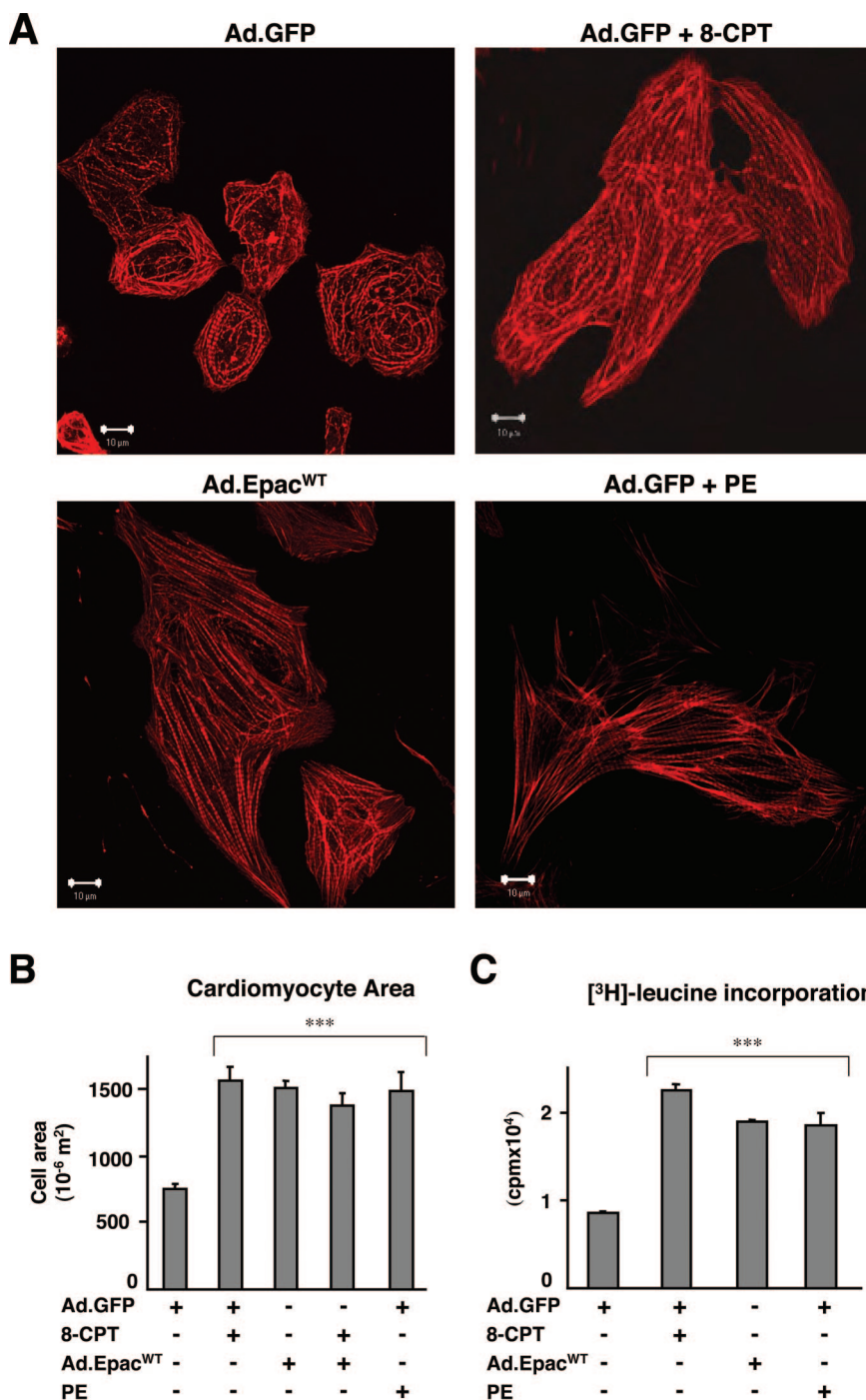
cell size and sarcomeric organization. Cardiomyocyte treatment with Ad.GFP and 8-CPT as well as infection of cardiomyocytes with Ad.Epac<sup>WT</sup> induced an apparent increase of the F-actin meshwork and a heavily striated appearance, reflecting the organization of this F-actin cytoskeleton into sarcomeric structures, as compared with cardiomyocytes infected with Ad.GFP alone (Figure 3A). Cells overexpressing Epac were hypertrophied and were not contaminated by fibroblast as shown by the  $\alpha$ -actinin staining in supplementary Figure I. In addition, the effects of Epac on sarcomeric organization were comparable to Ad.Epac- $\Delta$ cAMP (data not shown) and PE (Figure 3A), a well-known inducer of cardiac hypertrophy.

Next, we measured the effect of Epac on cell surface area. Activation of endogenous Epac with 8-CPT produced a two-fold increase in cell surface area when compared with cells infected with control Ad.GFP (Figure 3B). Identical results were obtained when cardiomyocytes were infected with Ad.Epac<sup>WT</sup> (Figure 3B), Ad.Epac- $\Delta$ cAMP (data not shown), or Ad.GFP and treated with PE (Figure 3B). The effect of Ad.Epac<sup>WT</sup> on cell surface area was not further increased in the presence of 8-CPT suggesting that basal intracellular cAMP was sufficient to activate recombinant Epac to induce its maximal effect on protein synthesis (Figure 3B). Finally, the effect of Epac on protein synthesis was analyzed by measurement of [<sup>3</sup>H]-leucine incorporation into cardiac myocytes. Expression of this cAMP-GEF resulted in an increase in [<sup>3</sup>H]-leucine uptake into cardiomyocytes (Figure 3C). Similarly, cell treatment with 8-CPT or the gold standard, PE resulted in an approximately two-fold increase in protein synthesis (Figure 3C). Altogether, these results show that Epac activation confers to primary cardiomyocytes all the features of the hypertrophic phenotype.

### Intracellular Ca<sup>2+</sup> Is Involved in Epac-Dependent Rac Activation

Alterations in intracellular Ca<sup>2+</sup> handling progressively exacerbate a hypertrophic or cardiomyopathic phenotype, in part

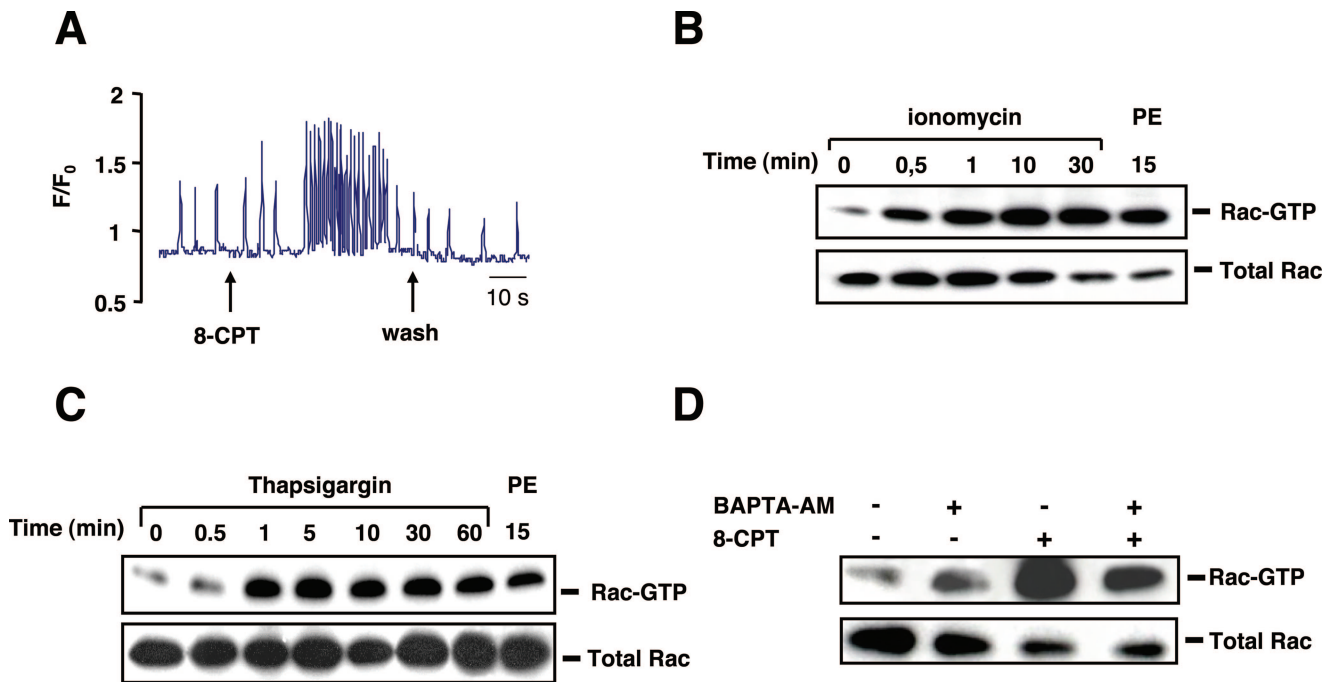




**Figure 3.** Epac induces cardiomyocytes hypertrophy. A, Fluorescent microscopic analyses of the effects of Epac on sarcomeric organization. Morphology of representative myocytes 48 hours after infection with Ad.GFP as a control, or Ad.Epac<sup>WT</sup> is shown. The Epac selective activator, 8-CPT was used at 10<sup>-6</sup> mol/L for 2 days in cells infected with Ad.GFP. Positive control cells were infected with Ad.GFP and treated with PE (10<sup>-5</sup> mol/L) for 2 days. Actin filaments were visualized by using Rhodamin-conjugated phalloidin. B, Photographic images of cells treated as above were digitized. The areas (10<sup>-6</sup> m<sup>2</sup>) of 30 to 50 individualized cells per condition from 2 to 3 independent experiments were determined by computer-assisted planimetry. Values show the means±SEM. C, [<sup>3</sup>H]-leucine incorporation. Cardiomyocytes were treated as in (A) and total radioactivity of incorporated [<sup>3</sup>H]-leucine into proteins was determined by scintillation counting. The figure shows the mean±SEM of data for 3 experiments performed in duplicate. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001 compared with control Ad.GFP.

through sustained activation of Ca<sup>2+</sup>-sensitive signal transduction pathways.<sup>19</sup> Given the involvement of Epac in cardiac hypertrophy, we examined whether its activation could affect intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in neonatal myocytes (Figure 4). At physiological external [Ca<sup>2+</sup>], cardiac myocytes exhibited spontaneous Ca<sup>2+</sup> transients with a low frequency (0.120±0.015 Hz, n=20) (Figure 4A). Application of the Epac agonist 8-CPT triggered a dramatic increase in the frequency of these Ca<sup>2+</sup> oscillations (0.51±0.04 Hz, n=7) without changing the amplitude of the spikes. This effect was also observed at 100 nM 8-CPT (0.40±0.05 Hz, n=13, data not shown).

Because Epac induced Rac activation, we examined the dependence of Rac activation on Ca<sup>2+</sup> signaling. Treatment of cardiac myocytes with the Ca<sup>2+</sup> ionophore ionomycin as well as an inhibitor of the Ca<sup>2+</sup>-ATPase, thapsigargin increased Rac activation in a time dependent manner (Figure 4B and 4C). The effect of ionomycin and thapsigargin on Rac activation was as potent as the positive control, PE (Figure 4B and 4C). Pretreatment with BAPTA-AM, an intracellular Ca<sup>2+</sup> chelator, attenuated Epac-induced Rac activation (Figure 4D). From these results we conclude that elevation of intracellular [Ca<sup>2+</sup>]<sub>i</sub> after Epac activation is sufficient to activate Rac.



**Figure 4.** Intracellular  $\text{Ca}^{2+}$  is involved in Epac-dependent Rac activation. A, Effect of 8-CPT ( $10^{-5}$  mol/L) on spontaneous spiking activity at  $1.8 \times 10^{-3}$  mol/L external  $[\text{Ca}^{2+}]$ . Cardiomyocytes at day 1 or 2 after plating were loaded with the  $\text{Ca}^{2+}$  indicator Fluo3-AM and perfused with a control external Ringer solution. B to D, Effect of ionomycin, thapsigargin and BAPTA-AM on Rac activation. Ventricular cardiomyocytes were treated at 2 days in vitro with either ionomycin ( $10^{-6}$  mol/L) (B) or thapsigargin ( $0.2 \times 10^{-6}$  mol/L) (C) for different times of incubation or PE ( $10^{-5}$  mol/L) for 15 minutes. D, Cells were pretreated with BAPTA-AM ( $1.5 \times 10^{-5}$  mol/L) for 10 minutes and then they were stimulated with 8-CPT ( $10^{-5}$  mol/L) for 15 minutes. The amount of Rac-GTP was determined by pull-down experiments.

### Epac Activates the Hypertrophic Calcineurin/NFAT Signaling Pathways

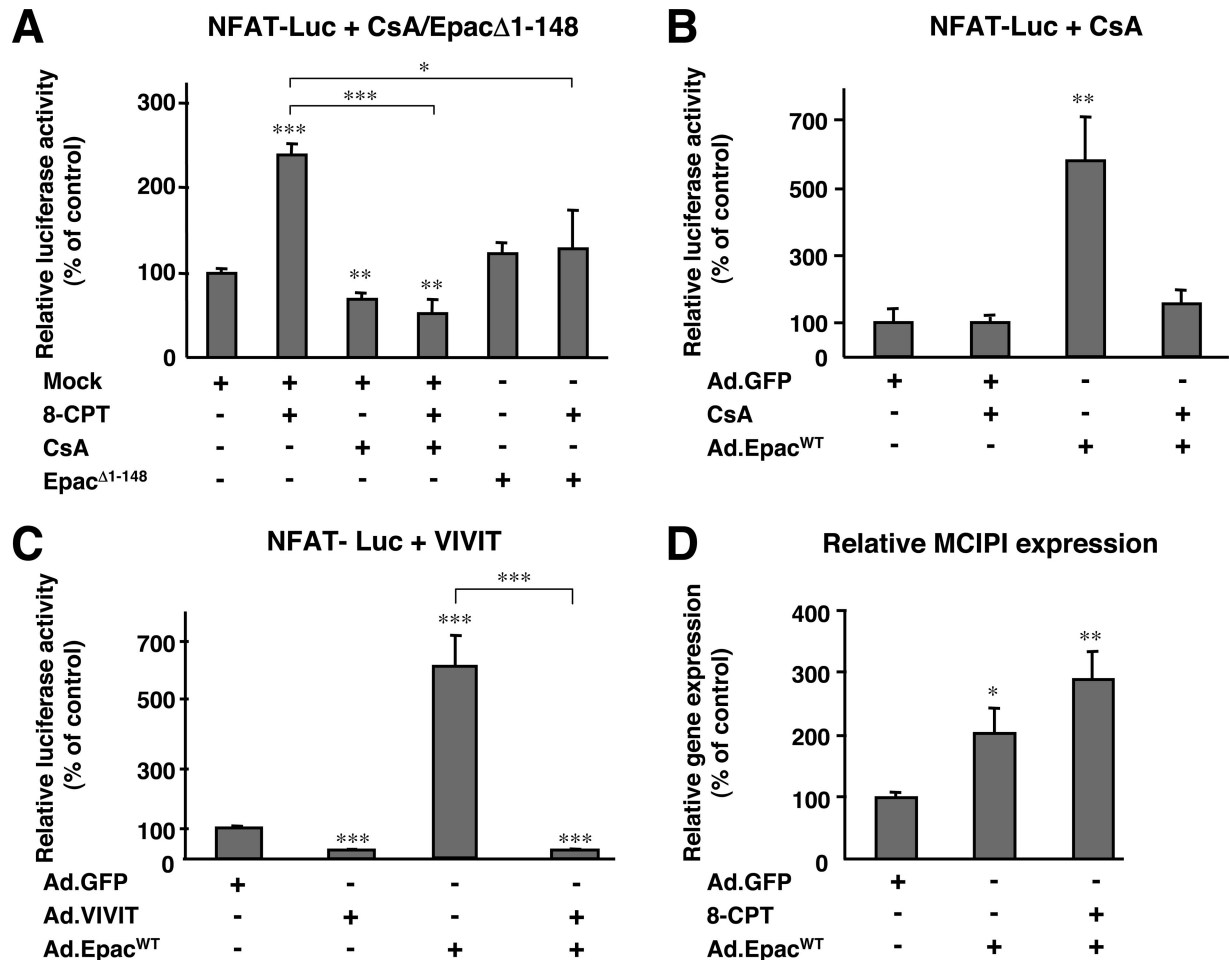
One prominent  $\text{Ca}^{2+}$ -dependent pathway that plays a crucial role in cardiomyocyte hypertrophy involves the phosphatase calcineurin.<sup>20</sup> Activation of calcineurin by  $\text{Ca}^{2+}$  results in the dephosphorylation and nuclear translocation of cytoplasmic NFAT transcription factors, which then upregulate transcription of hypertrophic genes. To test whether endogenous Epac may activate the hypertrophic calcineurin NFAT signaling pathway, cardiomyocytes were transfected with NFAT-Luc and treated or not with 8-CPT (Figure 5A). 8-CPT significantly increased NFAT transcriptional activity as compared with control cells (Figure 5A). Accordingly, 8-CPT increased NFAT nuclear translocation (supplementary Figure II). The stimulating effect of 8-CPT on NFAT-Luc was significantly blocked by a pharmacological inhibitor of calcineurin, cyclosporine A (CsA) or transfection of a dominant negative form of Epac (Epac $\Delta$ 1 to 148)<sup>21</sup> (Figure 5A).

Recombinant Epac<sup>WT</sup> also increased NFAT transcriptional activity which was blocked by CsA or an adenovirus bearing a selective peptide inhibitor of calcineurin named VIVIT (Ad.VIVIT)<sup>22</sup> (Figure 5B and 5C). Consistent with these findings, cardiac myocytes infected with Ad.Epac<sup>WT</sup> and treated or not with 8-CPT (Figure 5D), or Ad.Epac- $\Delta$ cAMP (data not shown) had an increased content of mRNA encoding the modulatory calcineurin-interacting protein 1 (MCIP1), a mediator of calcineurin signaling during cardiac hypertrophy.<sup>23</sup> Furthermore, coinfection with Ad.VIVIT and

Ad.Epac<sup>WT</sup> reduced the enhancement of sarcomeric organization and cell surface area induced by Ad.Epac<sup>WT</sup> (Figure 6A and 6B). Altogether these data show that NFAT is a downstream component of Epac hypertrophic signaling pathway.

### Involvement of Rac in Epac-Induced NFAT-Dependent Cardiomyocyte Hypertrophy

Because Rac was found to be a downstream component of Epac signaling pathway (Figure 1), we next examined the involvement of Rac in Epac-induced NFAT transcriptional activity. Ad.Rac<sup>S17N</sup> completely inhibited Epac-induced NFAT transcriptional activity (Figure 7A) whereas the stimulating effect of Rac<sup>G12V</sup> on NFAT-Luc was blocked by CsA (Figure 7B). These data clearly indicate that Rac is able to influence the calcineurin/NFAT signaling pathway. The involvement of Rac in Epac signaling pathway controlling cardiomyocyte hypertrophy was further supported by the observation that Rac<sup>S17N</sup> inhibited the stimulating effect of endogenous Epac activation or Epac<sup>WT</sup> on ANF expression (Figure 7C and supplementary Figure III). Consistent with these findings, Ad.Rac<sup>S17N</sup> inhibited Epac-induced cytoskeletal reorganization (Figure 7D) and increase in cell surface area (Figure 7E). Finally, as Rac has been shown to induce the production of reactive oxygen species (ROS),<sup>24</sup> we analyzed the effect of Epac<sup>WT</sup> on ANF-Luc in the presence of the antioxidant, N-acetylcysteine (NAC). We found that NAC inhibited Epac-induced ANF-Luc activity suggesting that oxidative stress is increased by Epac (supplementary Figure IV).



**Figure 5.** Activation of NFAT by Epac. A to C, Effect of Epac on NFAT transcriptional activity. A, Cardiomyocytes were transfected with NFAT-Luc, Epac- $\Delta$ 1 to 148, or the empty vector (mock) as control and treated or not with 8-CPT ( $10^{-6}$  mol/L) or CsA ( $0.5 \times 10^{-6}$  mol/L). B and C, Cardiomyocytes infected with Ad.GFP (control), Ad.Epac<sup>WT</sup>, or Ad.VIVIT were transfected with NFAT-Luc and treated as described. Two days after transfection, Luc activity was assayed. D, Epac increases MCIP1 expression. Cardiomyocytes infected with Ad.GFP (control) or Ad.Epac<sup>WT</sup> were treated or not with 8-CPT ( $10^{-6}$  mol/L) for 2 days. The ratio of MCIP1/GCB mRNA was determined by quantitative PCR. Values are expressed relative to the MCIP1/GCB ratio. Results were normalized to control for each experiment, and were expressed as means  $\pm$  SEM of at least 3 independent experiments performed in triplicate (A to C) or duplicate (D).

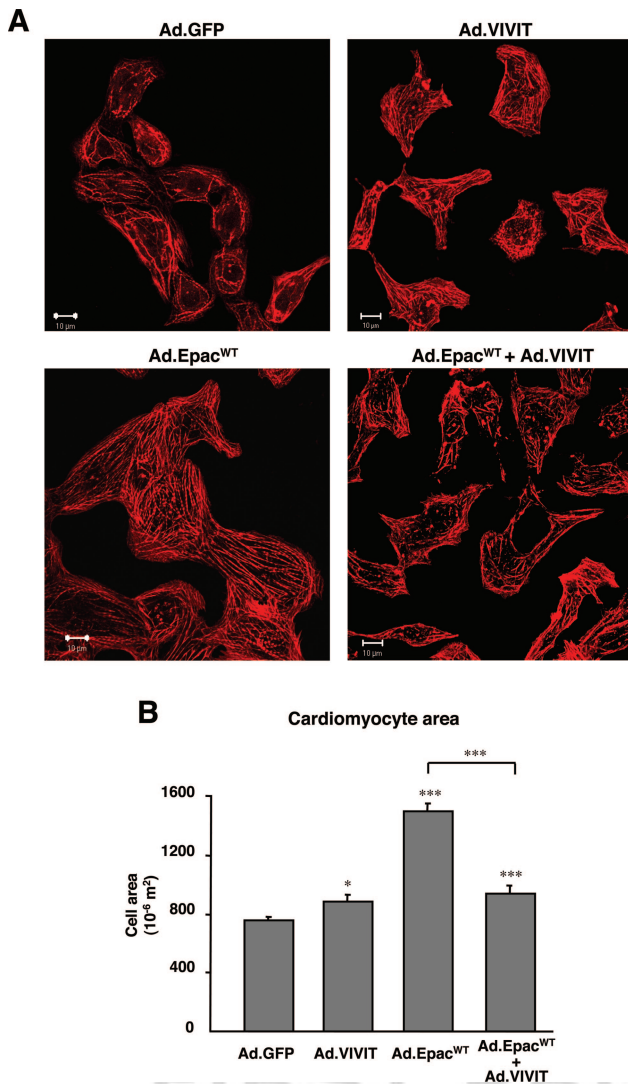
## Discussion

The present study shows for the first time that cAMP-dependent activation of Epac induces cardiomyocyte hypertrophy. This is based on our observation that Epac activation leads to morphological changes, cytoskeletal reorganization, increases in protein synthesis, and induces expression of cardiac hypertrophic markers. In addition, we found that Epac activates a prohypertrophic signaling pathway which involves calcineurin and its primary downstream effector, NFAT. Epac-induced NFAT activation was dependent on Rac activity. Interestingly, overexpression of Epac was able to influence cardiomyocyte morphology without cAMP analogue treatment indicating that the level of intracellular cAMP was sufficient to activate the recombinant Epac<sup>WT</sup> protein. Similar observations have been reported in other cellular systems because transfection of Epac<sup>WT</sup> in HEK293 and COS cells has been previously shown to influence cell signaling and morphology at resting basal cAMP levels.<sup>21</sup> Because Epac needs micromolar concentration of cAMP to be activated,<sup>4</sup> these

findings suggest that this cAMP-GEF may be localized in subcellular membrane fractions of cardiomyocytes where intracellular cAMP concentration is high. Further experiments are required to determine the precise localization of Epac in cardiac myocytes and its association with proteins (ie, phosphodiesterases) that regulate cAMP gradient formation.

In our study, we showed that the Epac-specific cAMP analogue 8-CPT produces bursts of  $\text{Ca}^{2+}$  transients in neonatal cardiac myocytes. Our findings are in line with recent studies in pancreatic  $\beta$ -cells and INS-1 insulin-secreting cells, demonstrating a Epac-dependent mobilization of intracellular  $\text{Ca}^{2+}$  by the cAMP-elevating hormone glucagon-like peptide 1 and the implication of Epac in this effect.<sup>25</sup> In these cells, activation of endogenous Epac triggers  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release<sup>26</sup> and it is suggested that a functional coupling exists between Epac and the RyR in these cellular systems.<sup>27</sup> Interestingly, the small GTPase Rap1 which is an effector of Epac is suspected to play a role in cAMP-induced  $[\text{Ca}^{2+}]_i$  increase via SERCA3b in megakaryocytes.<sup>28,29</sup> Therefore,





**Figure 6.** Ad.VIVIT inhibits Epac-induced cardiomyocyte hypertrophy. A, Fluorescent microscopic analyses of the effects of Epac on sarcomeric organization. Morphology of representative myocytes 48 hours after infection with Ad.GFP (control), Ad.VIVIT, Ad.Epac<sup>WT</sup>, or Ad.Epac<sup>WT</sup> and Ad.VIVIT is shown. B, Photographic images of cardiac myocytes infected as above were digitized. Areas (10<sup>-6</sup> m<sup>2</sup>) of around 50 individualized cells per condition from 3 independent experiments were determined by computer-assisted planimetry. Values show the means ± SEM. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 compared with control or vs indicated values.

one could imagine in cardiac myocytes, that Epac might interact with Ca<sup>2+</sup> release channels. Such hypothesis is currently undergoing investigation.

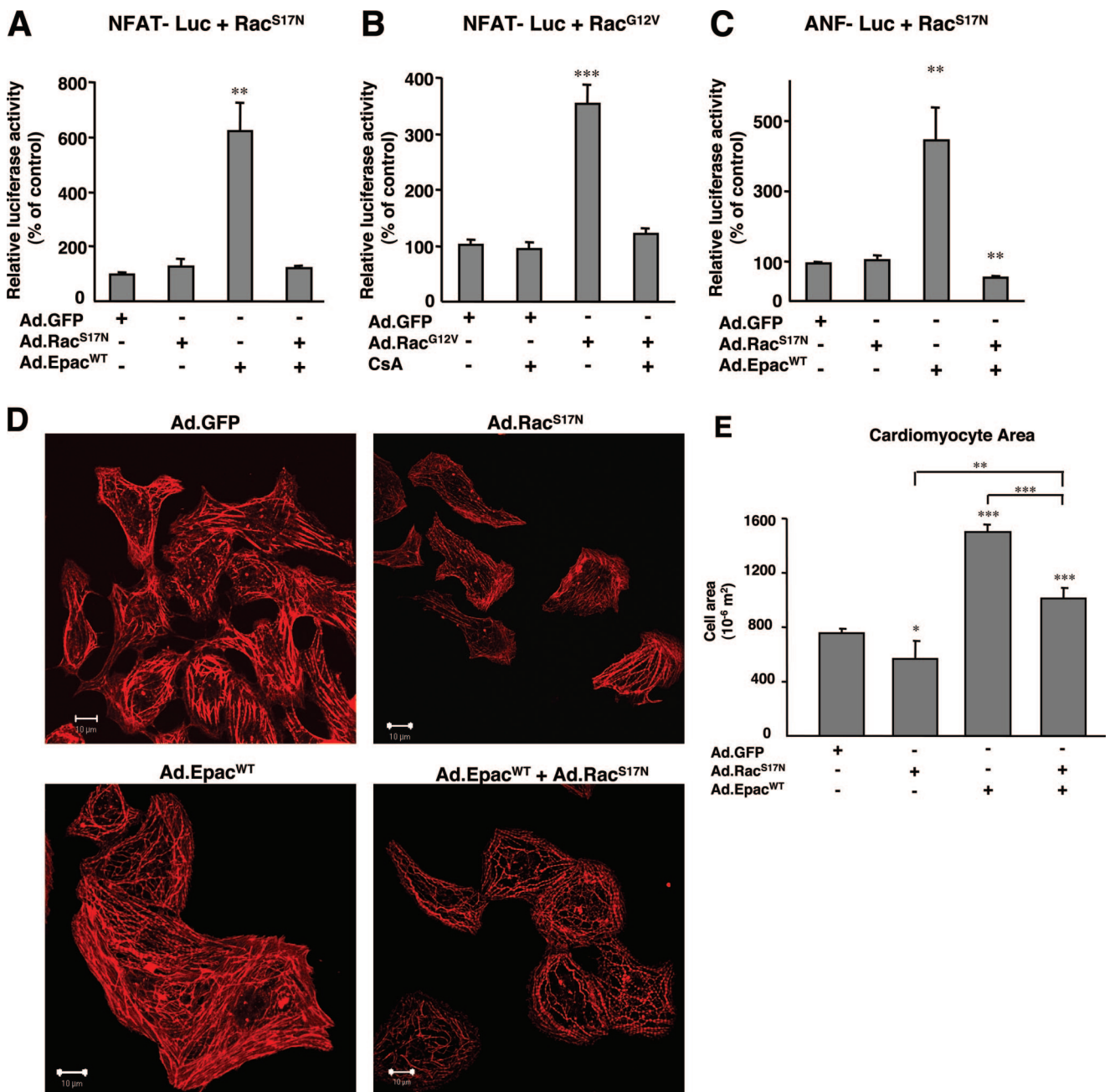
We found that Epac induced Rac activation in primary cardiomyocytes. This is in accordance with our recent findings showing that Epac induces Rac activation in a cAMP-dependent but PKA-independent manner in noncardiac cells such as primary cortical neurons and CHO cells.<sup>11</sup> Because we found that Rac was activated by Ca<sup>2+</sup> on Epac stimulation, it is reasonable to think that Rac might be regulated by a GEF, which is sensitive to Ca<sup>2+</sup>. Such a GEF has been reported for small GTPases of the Ras family.<sup>30,31</sup> Another molecular

target which could be involved in Ca<sup>2+</sup>-dependent Rac activation is the Rho GDP-dissociation inhibitor (RhoGDI). RhoGDI retains Rac into the cytoplasm and must dissociate to allow Rac to encounter its GEFs.<sup>32,33</sup> Recently, Price et al<sup>34</sup> have shown that Ca<sup>2+</sup> induces a disruption of the Rac-RhoGDI complex leading to the translocation and activation of Rac in PC3 cells. Thus, one could speculate that such a mechanism might occur in cardiomyocytes and contribute to Epac-induced Ca<sup>2+</sup>-dependent Rac activation

We report for the first time to our knowledge that Epac is implicated in the activation of NFAT in cardiac myocytes. The ability of Epac to stimulate NFAT activity was significantly inhibited by treatment with CsA and VIVIT, suggesting that calcineurin activity is regulated by Epac. Accordingly, we found that Epac upregulates the expression of MCIP1, a well known modulator of calcineurin signaling that possesses a series of NFAT binding sites in its gene promoter.<sup>23,35</sup> In addition, Ad.VIVIT partially reversed Epac-induced cardiomyocyte hypertrophy indicating that Epac is a new regulator of the hypertrophic calcineurin/NFAT signaling pathway. As the Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) is well-known to play a key role in cardiac hypertrophy,<sup>36</sup> it would be interesting to test the potential involvement of this signaling pathway in Epac-induced cardiac hypertrophy. In addition, Epac might be an important mediator of oxidative stress because an antioxidant blocked its effect on ANF-Luc. In accordance with this observation, Rac activation is thought to be an important mediator of ROS production induced by adrenoceptor stimulation.<sup>37</sup> Furthermore, G<sub>α(12/13)</sub>-mediated ROS production is essential for angiotensin II-induced NFAT transcriptional activation.<sup>24</sup>

An important finding of the present study is that the effect of Epac on NFAT activation was inhibited by Rac<sup>S17N</sup>, a negative dominant form of Rac. Inversely, Rac under its activated form increased NFAT activity and this effect was blocked by CsA. In line with these data, we found that Rac<sup>S17N</sup> inhibited Epac-induced ANF transcriptional activity and cell growth. Altogether these results indicate that Rac is involved in the regulation of the hypertrophic calcineurin/NFAT signaling pathway initiated by Epac in cardiomyocytes. In contrast to our findings, a previous study has shown that Ras but not Rho GTPases regulates NFAT activity in cardiac cells.<sup>38</sup> The reasons for these discrepancies are still unclear. However, the stimulating effect of Rac on NFAT activity is supported by previous reports showing that humoral factors induce Rac-dependent NFAT activation in various cellular systems including immune and cardiac cells.<sup>24,39,40</sup> In addition, Rac<sup>G12V</sup> has been shown to upregulate ANF expression in rat primary cardiac myocytes.<sup>14</sup>

Besides Epac, sustained activation of other cAMP effectors have been shown to be deleterious for cardiac cells. For instance, constitutive activation of PKA in the hearts of transgenic mice leads to cardiomyocyte hypertrophy and a progressive decline in cardiac function.<sup>41</sup> In a similar manner, increasing β<sub>1</sub>-adrenergic receptor (β<sub>1</sub>-AR) signaling cascade or G<sub>αs</sub> protein levels induces, through intracellular Ca<sup>2+</sup> elevation, a progressive development of cardiac hypertrophy and heart failure.<sup>42,43</sup> But although our data and the these observations point to a negative role of persistent activity of



**Figure 7.** Involvement of Rac in Epac-induced NFAT-dependent cardiomyocyte hypertrophy. A to C, Cardiomyocytes infected with Ad.GFP (control), Ad.Rac<sup>S17N</sup>, Ad.Epac<sup>WT</sup>, or Ad.Epac<sup>WT</sup>, and Ad.Rac<sup>S17N</sup> were transfected with either NFAT-Luc or ANF-Luc. Two days after, Luc activity was determined. Values are means $\pm$ SEM of at least 3 separate experiments performed in triplicate. D and E, Ad.Rac<sup>S17N</sup> reverses Epac-induced cardiomyocyte hypertrophy. D, Photographic images of cells infected for 2 days with Ad.GFP (control), Ad.Epac<sup>WT</sup>, Ad.Rac<sup>S17N</sup>, or Ad.Epac<sup>WT</sup>, and Ad.Rac<sup>S17N</sup> were digitized. E, The areas (10<sup>-6</sup> m<sup>2</sup>) of 50 individualized cells per condition from 3 independent experiments were determined by computer-assisted planimetry. Values show the means $\pm$ SEM. \* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001 vs control cells or indicated values.

cAMP/Epac/PKA, any elevation of cAMP does not automatically cause deleterious effects. For instance, transgenic mice overexpressing  $\beta_2$ -AR in the heart,<sup>44</sup> Adenyl cyclase type 6 (AC6)<sup>45</sup> or AC8<sup>46</sup> do not show early signs of hypertrophy or heart failure. Clearly, these data show that the same second messenger conveys different information and cAMP compartmentation is a key actor and determines the quality of the response. As a next step, it therefore will be crucial to determine not only the spatial localization of Epac and its possible interaction with cAMP-PDE but also the neurohor-

monal factors which are involved in the regulation of its activity.

Thus, we propose a new cAMP signaling pathway in which activation of Epac leads to an increase in [Ca<sup>2+</sup>]<sub>i</sub>, which then activates calcineurin and Rac. The latter controls NFAT activation. This signaling cascade activates hypertrophic gene expression and induces the morphological aspects of cardiac myocyte hypertrophy. Our results thus open new insights into the signaling pathways by which cAMP may mediate its biological effects in cardiomyocytes.

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